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13. ABSTRACT *(Maximum 200 words)*

Mac25 is the seventh member of the IGFBP family (IGFBP-7). IGFBP-7 is expressed in normal human mammary epithelial cells (HMECs) and upregulated in senescent HMECs. IGFBP-7 is not expressed in twelve of sixteen breast cancer cell lines, in studies by two laboratories. Its expression is absent in estrogen receptor positive (ER^+) breast cancer cell lines.

IGFBP-7 may regulate HMEC growth. We will test the hypothesis that it suppresses growth in ER^+ breast cancer cells. We will also test the ability of IGFBP-7 to negatively affect growth rates in ER^+ breast cancer cells by transducing the cDNA into normal and human breast tumor cultures using a retroviral vector system. We will determine if IGFBP-7 is regulated at the transcriptional level by characterization of the promoter region of the gene. We will determine binding properties of IGFBP-7 protein by gel shift analysis of protein extracts and anti-IGFBP7 antibody. We will determine if IGFBP7 is hormonally regulated by studying tissue specificity of RNA expression and protein localization through *in situ* hybridization and immunohistochemistry (additional aim).

We have re-evaluated the sequence for IGFBP-7 (mac25) by PCR/sequence analysis of cDNA made from five cell lines. Two of ten clones obtained from a library screening contain sequence upstream of the IGFBP-7 gene and may contain promoter region. We have assessed two IGFBP-7 antibodies. Differences of mRNA expression were found between male and female C57BL6 mice.

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Karen Swisshelm 9/18/97
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September 18, 1997

Mac25 (IGFBP-7), a new insulin-like growth factor binding protein and its role in breast cancer
Mentor: Karen L. Swisshelm, Ph.D.
Trainee: Heather-Marie P. Wilson

attached cover letter, 1 copy, unnumbered

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Mentor: Karen L. Swisshelm, Ph.D.
Trainee: Heather-Marie P. Wilson

Mac25 (IGFBP-7), a new insulin-like growth factor binding protein and its role in breast cancer

INTRODUCTION

Background

Cell growth control encompasses an interplay between autocrine, paracrine, and endocrine growth factors; their receptors; and the complex set of molecules that regulate factor/receptor action, including the extracellular matrix and stromal microenvironment. One set of factors important for growth of mammary epithelial cells are the insulin-like growth factors (IGFs) (1, 2). IGFs are circulating peptides that initiate signal transduction by binding to a transmembrane insulin-like growth factor receptor (IGFR). To date, two IGFs have been characterized: IGF-I and IGF-II. Both IGFs have a structure homologous to proinsulin and are potent mitogens in many cultured cell lines, including the breast cancer cell line, MCF-7 (3). Two IGFRs have been documented: type 1 IGFR and type 2 IGFR. Type 1 IGFR has a structure that is homologous to the insulin receptor.

Interaction between growth factors, such as the IGFs, and compatible receptors is frequently modulated by another molecule that binds to the growth factor and "titrates" ligand/receptor interaction. One such class of molecules is the insulin-like growth factor binding protein (IGFBP) family. IGFBPs complex with IGFs and thereby control IGF interaction with the IGFRs, or other potential receptors. At present, there are at least seven IGFBPs, including mac25 (now referred to as IGFBP-7) (4). IGFBPs may be cleaved within the extracellular matrix (ECM). Thus, IGFBP peptides may have other roles besides growth factor interaction, such as activation/inactivation of other cellular receptors.

Identification of mac25/IGFBP-7/PSF by Different Strategies

Mac25/IGFBP-7 was first identified in a plasmid subtraction library screen from a meningioma cell line (5). Those investigators showed that mac25 mRNA expression was inversely correlated with estrogen receptor positivity in a panel of human breast cancer cell lines. Sequence analysis indicated that mac25 was a member of the IGFBP family based on the strong homology at the amino terminus of mac25/IGFBP-7 and several members of

the IGFBP family. Mac25/IGFBP-7 was found during a study designed to uncover genes overexpressed in normal senescent human mammary epithelial cells (6). Sequence analysis showed the gene to be the same as that found by Murphy *et al.* The gene was mapped to chromosome 4q12 by FISH. Cytogenetic deletions in an adjacent region, 4q13, have been observed in primary breast cancer (7).

In an attempt to purify and clone a prostacyclin-stimulating factor (PSF), mac25/IGFBP-7 was recovered from cultured human diploid fibroblast cells (8). This was achieved through a series of protein purification steps, including an insulin-like growth factor-I ligand affinity column. By SDS/PAGE, PSF was found to run as a 31 kDa molecular mass. The amino acid sequence of the purified protein was determined by automated Edman degradation and used to derive a pair of degenerate primers. With these primers, a 59 nucleotide fragment was generated by RT-PCR. A pair of primers was designed from this fragment to screen two libraries and sequence the cDNA inserts. In an independent study to determine extracellular matrix-degrading proteinases and their inhibitors during tumor invasion and metastasis, a 30 kDa protein was purified, which demonstrated cell adhesion activity, referred to as tumor-derived adhesion factor (TAF)(9). Subsequent papers show 100% identity between TAF and PSF and the authors suggest that TAF and mac25/IGFBP-7 may be the same molecule (see Figure 2)(10, 11).

Another study suggests that mac25/IGFBP-7 also shares homology with follistatin, an activin-binding protein (12). Sequence comparisons revealed that mac25/IGFBP-7 contained regions highly conserved in follistatin (see Figure 2). A missing carboxy terminus indicates that mac25/IGFBP-7 may be a product of alternative splicing and behave as a truncated follistatin, having stronger activin-binding properties than the untruncated version. Using murine mac25, Kato *et al.* demonstrated inhibition of a p53-deficient osteosarcoma cell line, indicating that mac25/IGFBP-7 may be a tumor suppressor that mediates activin, a member of the TGF-beta superfamily.

In recent studies, human recombinant (hR) mac25/IGFBP-7 protein of 27 kDa was generated using a baculovirus expression system (4). The investigators demonstrated hR-mac25 specifically interacts with IGF-I and IGF-II by Western ligand blotting, indicating that mac25 is a member of the IGFBP family. This same group generated an antibody against the hR-mac25 in rabbit (13). In these studies, a 31 kDa protein was detected in the conditioned medium of Hs578T (an ER^- breast cancer cell line), human sera, amniotic

fluid, cerebral spinal fluid (CSF), and urine. *Mac25 is now referred to as IGFBP-7 in literature and the same will be done in this review.*

The above studies suggest that IGFBP-7 is found in a wide range of tissues. Because of its higher expression level in senescent cells and lower mRNA expression in ER^+ breast cancer cell lines (6), it is likely that IGFBP-7 has growth-suppressing capabilities in addition to its ability to bind IGF-I and IGF-II. IGFBP-7 may be important in cell growth regulation not only in breast tissue, but in other tissues where mRNA/protein expression is present.

Hypothesis/Purpose

IGFBP-7 is a gene with high homology to a hormone/growth factor binding protein in the IGFBP family that weakly binds IGF-I and IGF-II. We hypothesize that it may suppress the growth of ER+ breast cancer cells. IGFBP-7 may be transcriptionally regulated by retinoids and estrogen.

Scope/Technical Objectives

To test the hypotheses presented above, I propose the following studies.

Specific Aims

Aim 1. We will test the potential of IGFBP-7 *function* as a negative effector of cellular growth rates in ER^+ breast cancer cells. We propose to transduce the cDNA into normal and human breast tumor cultures using a retroviral vector system.

Aim 2. We will test the hypothesis that IGFBP-7 is *regulated* at the transcriptional level by retinoic acid through a retinoic acid response element and by estrogen through an estrogen response element via cognate receptors. We propose to characterize the mac25 promoter.

Aim 3. We will determine protein binding *characteristics* of IGFBP-7 using purified IGFBP-7 protein and a specific antibody.

Aim 4 (Additional). We will determine tissue specificity of RNA expression and protein localization by in situ hybridization using tissues collected from C57BL6 mice. We began this last aim in order to determine if IGFBP-7 was *hormonally* regulated in mice.

BODY

Aim 1: Experiments to address the potential of IGFBP-7 for modulating cell growth of ER^{+} breast cancer cells.

Experimental Methods

The full length cDNA of IGFBP-7 (mac25) has been cloned from a normal HMEC lambda Zap cDNA library, as verified by restriction mapping. Subsequently, the cDNA has been inserted into the BamHI site (Ryan, K., Sager, R., and Swisshelm, K., unpublished) of an expression vector containing a cytomegalovirus (CMV) promoter as well as the gene for the selectable marker for neomycin (vector from B. Vogelstein, unpublished).

For transfection experiments, we will use a retroviral vector system to test the effect of IGFBP-7 expression in breast cancer cell lines lacking expression, using the pLXSN vector (14). Our original design was to use the tetracycline repressor/operator plasmid system, an inducible system. The retroviral vector offers several advantages: primarily, it incorporates a single copy of the gene of interest per cell, which may give a more accurate idea of how constitutive IGFBP-7 expression would affect cell growth.

The pCMV-neo vector containing the full-length IGFBP-7 cDNA was digested with BamHI to confirm the insert size, ~900 base pairs. XL1-Blue *E.coli.* were transformed with the vector for amplification purposes, and the plasmid was isolated by plasmid midiprep (Qiagen). Two micrograms of the vector was digested with BamHI to check for insert size. Upon confirmation, ~16 micrograms DNA was digested and the IGFBP-7 construct was isolated on a 1% agarose gel and eluted by centrifugation (Ultrafree-MC, Millipore, Bedford, MA). The full-length construct was ligated into pBluescriptII-KS (confers ampicillin resistance) and transformed into XL1-Blue *E.coli.* The plasmid (pBl-mac25) was isolated and digested with BamHI to verify insert size. The IGFBP-7 construct was sequenced by automated and manual procedures, taking advantage of the primer sites present in the pBluescriptII-KS.

The full-length IGFBP-7 construct was ligated into the retroviral vector LXSN (14). This system relies on use of retrovirus-packaging cell lines, in the absence of helper virus, to aid in producing replication-defective retroviral vectors (15, 16). The LXSN vector contains long terminal repeats (L), a cloning site (X), a simian virus 40 early promoter (S), and a neomycin resistance gene that allows G418 selection (N). This vector contains two promoters. One promoter allows expression of the neomycin gene. The second promoter, the retroviral LTR is responsible for transcription of the inserted cDNA. The vector also contains a psi+ region. This region is required for packaging viral RNA into virions and allow the retroviral vectors to obtain high titers of 10^6 to 10^7 colony forming units (cfu). The IGFBP-7 construct was ligated into the BamHI site and partially sequenced to determine orientation. The plasmid vector containing the sense and antisense orientations of mac25 were named pL*BP7*SN and pL*antiBP7*SN, respectively.

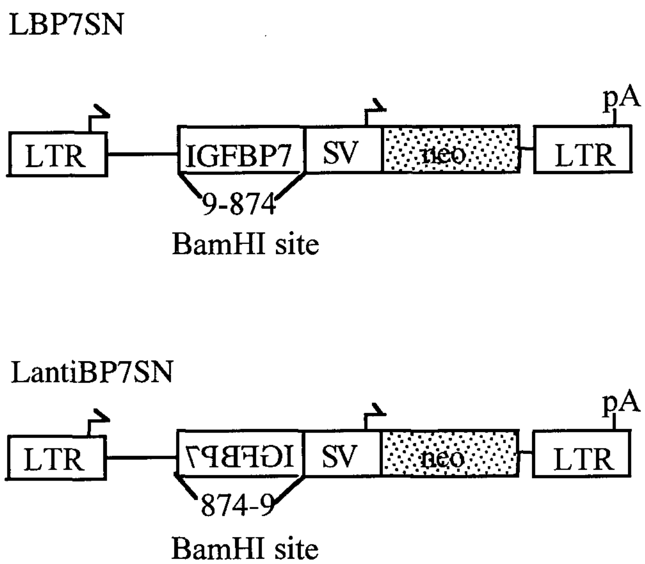

Figure 1. Maps of the retroviral vectors used to transduce cell lines of interest.

To generate stable vector-producing cell lines, PE501 cells were transfected with the L*BP7*SN (sense RNA) and L*antiBP7*SN (antisense RNA, control) by calcium phosphate coprecipitation. Virus generated from this cell line are capable of infecting cells from rat or mouse (ecotropic). After two days, the virus was harvested and used to infect PA317 cells, which have a wide host range, including human cells (amphotropic). The vector-infected PA317 cells were grown in the presence of G418 and 20 clones collected for each vector (pL*BP7*SN and pL*antiBP7*SN). This clones were checked for: unrearranged vector (digestion), vector titer, absence of helper virus, and expression of inserted gene

(Northern). The virus has been collected and used to infect the breast cancer cell lines of interest.

Results and Discussion

Sequencing

Sequencing studies on pBl-BP7 that the IGFBP-7 cDNA construct contains 865 nucleotides (nucleotides 9-874 in Genbank). There were six nucleotide deviations that resulted in six conservative amino acid changes (22/23CG -> GC, 44T -> C, 50C -> G, 291G -> A, 297G -> A, and 584C -> T) and one guanosine nucleotide "deletion" at position 830. The conserved amino acid changes could be due to polymorphism. Originally, we thought the "deletion" was likely to be the result of a cloning error that occurring during replication or moving the insert between plasmid vectors.

At this point, it was decided that several cell lines should be screened and checked for the absence or presence of the guanosine deletion. Four cell lines were used: AG11132, AG11134, MDA-MB-231, and SKBR3. RNA isolated from these cell lines were reverse transcribed using reverse transcriptase, M-MuLV (Boehringer Mannheim, Germany). The resulting cDNA was amplified by PCR using primers that surround nucleotides 752-898, the region containing the deletion. The resulting amplified cDNA was treated with shrimp alkaline phosphatase and exonuclease I to remove unused primers and inactivate enzymes. A portion of the PCR reaction sample was used directly for sequencing (Sequenase, USB).

The sequences obtained from the four cell lines match the sequences found in the pBl-BP7 construct and the 76N library. Therefore, it was concluded that our construct is the correct version and not the result of a cloning error. The resulting sequence is five amino acids longer and is identical to the sequences submitted for tumor-derived adhesion factor (TAF) and prostacyclin-stimulating factor (PSF). **[Figure 2]**

Southern

Genomic DNA was isolated from transduced PA317 cells containing L*BP7*SN or L*antiBP7*SN. Ten micrograms of the DNA was digested with SmaI, separated by gel electrophoresis, and transferred to Zeta Blot. The blot was probed with ^{32}P-labeled IGFBP-7, stripped, and reprobed with the neomycin gene. SmaI cleaves IGFBP-7 at position 674 and LXSN at positions 648 and 3398. The predicted band sizes detected with ^{32}P-labeled IGFBP-7 would be: L*BP7*SN - 1.68kb, 1.94kb, 3.62kb(uncut); L*antiBP7*SN - 1.21kb, 2.41kb, 3.62kb(uncut). Indigenous IGFBP-7 DNA would also

demonstrate hybridization to the probe. ^{32}P-labeled neomycin gene should detect: L*BP7*SN - 1.94kb, 3.62kb(uncut); L*antiBP7*SN - 2.41kb, 3.62kb(uncut).**[Figure 3, A and B]**

Northern

Total RNA was isolated from the same cell clones (transduced PA317 cells containing L*BP7*SN or L*antiBP7*SN) used above to determine expression of the vector. The RNA was separated by gel electrophoresis, transferred to Zeta Blot, and hybridized with IGFBP-7 and the neomycin gene. There are two promoter sites present in the vector (refer to figure 1). The first promoter site, located in the LTR region will yield a predicted transcript of 3.6kb. The second promoter (SV40) is located after the IGFBP-7 insert site. It should yield a transcript of 1.38kb. Total RNA was also collected from PA317 cells that were transduced with LXSN (no insert). It should show two transcripts of 1.34kb and 2.75kb. It was difficult to distinguish retroviral RNA from indigenous RNA when hybridized with IGFBP-7. Hybridizing with the neomycin gene showed clearer results. **[Figure 3C]**

Conclusions

- New sequence for mac25/IGFBP-7 identified
- Retroviral vector, containing sense and antisense IGFBP-7, has been successfully incorporated into PA317 genome; the vector is intact and expression is detected.

Aim 2: Experiments for promoter studies; library screening and clone analysis.

<u>*Experimental Methods*</u>

A human chromosome 4 genomic library was screened for clones that hybridize with a probe for IGFBP-7 cDNA. Ten clones were obtained. These clones were analyzed by restriction digests, PCR and DNA-DNA hybridization of dot blots.

Single-strand DNA was isolated from lambda bacteriaphage collected from four of the ten clones (clones 1, 4, 5, and 9) through cell lysis by clone phage and PEG precipitation of resultant lysate, using a protocol that includes the following steps: RNAseA and DNAseA treatment, incubation with proteinase K, and phenol/chloroform extraction. This DNA was digested with HindIII and analyzed by gel electrophoresis. The gel revealed clones 4 and 5

to have inserts of approximately 5 kilobases. No detectable inserts were observed for clones 1 and 9.

PCR analysis was accomplished using various primers of IGFBP-7 sequence and two primers that surround the HindIII insert site of Charon 21A. The results are suggestive that we may have regions 5' of the IGFBP-7 cDNA. However, it is not clear if this region is actually promoter region or if it contains introns **[Figure 4]**.

To examine the clones in a more efficient manner, a dot blot containing lambda bacteriaphage from the ten clones was hybridized with probes which corresponded to the 5' end of IGFBP-7. Initial results indicate that clone 5 and 7 may contain the 5' portion of IGFBP-7, and perhaps some of the promoter region. However, the phage coat proteins seem be interfering and giving false signal through a high background. It was decided to repeat this experiment using purified DNA from the ten clones and reprobe with the 5' end of the IGFBP-7 sequence.

Conclusions

- Ten clones isolated from ATCC library enriched for chromosome 4.
- Two of four clones screened have region 5' of the IGFBP-7 cDNA.

Aim 3: Experiments to determine binding characteristics of IGFBP-7 using purified IGFBP-7 protein and a specific antibody.

<u>Experimental Methods</u>

A short peptide sequence was designed using the translated IGFBP-7 cDNA sequence. This sequence, MECVKSRKRRRGKAGA: corresponding to amino acids 118-133 was chosen because of this regions high antigenic profile, by hydrophilic qualities using the rationale that hydrophilic regions of a protein are most likely to be located on the surface of the protein (Hopp and Woods Analysis) (17). The peptide sequence was also chosen from a region of the IGFBP-7 sequence having no homology with any of the other IGFBPs. The peptide was further examined by BlastP and FastA searches of the Gen EMBL-protein database to ensure this uniqueness. Research Genetics (Huntsville, Alabama) was used to generate the rabbit polyclonal antibodies. These polyclonal antibodies were not affinity purified and are in serum form.

The specificity of the rabbit antibodies was tested against IGFBP-7 by immunoblots using enhanced chemiluminescence for detection of IGFBP-7 in normal and breast cancer cell lines that do (AG11132 - normal, Hs578T - cancer) and do not (MCF7 - cancer) express IGFBP-7 mRNA.

Results from dot blots using only the two cancer lines (Hs578T and MCF7) gave ambiguous results. Therefore, it was decided to use Western gels to separate the proteins according to their molecular weights and then look for differences in antibody binding of the various bands, comparing the 8 week, 10 week, and 12 week bleeds to the pre-adjuvant serum collected from the two rabbits used (20673 and 20692). Results remained ambiguous.

Antibody made against human recombinant (hR) IGFBP-7 and purified hR IGFBP-7 (control) was obtained from the Dr. Ronald Rosenfeld laboratory, Oregon Health Sciences University, Portland, OR (4). This antibody was tested with anti-peptide antibodies. Results suggested that the 10 week bleed of the anti-peptide antibody may have some binding at 31 kDa corresponding to that seen with the IGFBP-7 antibody. However, subsequent gels revealed this band to be an artifact of the gel because it was observed in all lanes, including those with the molecular weight ladders or containing no sample.

Another problem encountered with the anti-peptide antibody was the small amounts of IGFBP-7 present which may have been too little to detect in conditioned medium. In a paper by Yamauchi *et al.* which describes the purification of PSF from serum-free conditioned medium of human diploid fibroblast cells, three liters of conditioned medium was used to obtain 1.2 micrograms of purified protein. This corresponds to approximately 4 picograms of IGFBP-7 in the 10 microliters of conditioned medium, implying we should be developing methods for concentrating protein or media samples.

Precipitation of the protein in the serum-free medium collected from cultured AG11132 (passage 10), Hs578T, and MCF7 cell lines was attempted in order to enrich the specific concentration of detectable IGFBP-7 protein. The cells were grown to near confluency in serum-containing media and washed twice with phosphate buffered saline (PBS). Serum-free media was added and collected at 24 hours. Two aliquots of 54 milliliters each was collected from each cell line. One aliquot was crosslinked with dissucinyl suberate (DSS) to determine if IGFBP-7 was bound to any proteins. This could be determined by seeing a band shift to a higher molecular weight when compared with the uncrosslinked sample.

Both aliquots were precipitated with 26.3 grams of ammonium sulfate at 4°C for an 80-85% saturation level. After the salt completely dissolved, the samples were centrifuged. The resulting pellet was resuspended in PBS/10% glycerol and dialyzed in PBS/10% glycerol overnight at 4°C. Excess salts were removed by centrifugation after dialysis. The ten microliters of sample loaded per well contains a predicted 432 picograms of IGFBP-7. This is a 100-fold increase from the amount of sample previously loaded.

Results and Discussion

Western blots using the samples collected above and hR IGFBP-7 were incubated with preimmune, 10 week, and 12 week bleeds of rabbit 20673 as well as anti-IGFBP7 from the Rosenfeld lab. Our results show a band present at approximately 50 kDa in the lanes containing Hs578T (uncrosslinked) and the hR IGFBP-7. There were no bands detected for the other samples. It is likely that AG11132 did not exhibit a detectable band because an early passage of the cells was used. IGFBP-7 mRNA is more abundant in *senescent* human mammary epithelial cells. This experiment will be repeated using AG11132 collected at passage 17 or 18 **[Figure 5]**. The detection of a >50kDa protein in comparison to the 31kDa shown in the work of Oh *et al.* (4) may be due to differences in our treatment of molecular weight markers. It is necessary to reduce the molecular weight markers with ß-mercaptoethanol before running them next to reduced protein samples. If this step is omitted, the molecular weight markers will run much slower than the reduced samples, giving the impression of sample proteins being smaller.

A 50kDa protein is highly likely as the IGFBP-7 protein sequence has multiple sites for protein modification, such as phosphorylation and glycosylation (6).

Conclusions

- We have an antibody that recognizes IGFBP-7.
- IGFBP-7 is 50kDa, indicating that is has been modified.

Aim 4 (Addition): Experiments to determine if tissue specificity is gender dependent for RNA expression/protein localization of IGFBP-7 in mice.

Experimental Methods

Isolation and preparation of RNA

Mouse Tissue. Tissue was collected from male and female C57BL6 mice, snap frozen in liquid nitrogen, and stored at -70^{0}C until use. Ultraspec-II RNA Isolation System (Biotexc, Houston, TX) was used to extract total RNA.
Cultured Cells. Cells were grown to confluency, followed by extraction using (guanidine-isothiocyanate and sodium citrate)-2-mercaptoethanol and ultracentrifugation in the presence of cesium chloride.

Northern Analysis
RNA samples of 10 micrograms each were solubilized and electrophoresed on a 1% denaturing agarose gel in the presence of ethidium bromide, transferred to Zeta Probe Blotting Membranes (Biorad, Hercules, CA), and UV crosslinked (UV Crosslinker, FisherBiotech). The membranes were probed with ^{32}P-labeled sic2.cl16 corresponding to the 3' end of mac25 (nts: 124-1096). After autoradiography, the membranes were stripped. The stripped blots were reprobed with ^{32}P-labeled 36B4 probe as a control for even loading of RNA.

Quantification has been performed using Adobe Photoshop, NIH Image, and Excel.

RNA In Situ Hybridization
Tissue Collection. Tissue was collected from ICR (outbred) and C57BL6 (inbred) mouse strains and frozen in O.C.T. freezing medium (Sakura Finetek, Torrance, CA) in isopentane (Sigma, St. Louis, MO) chilled over liquid nitrogen. Samples were stored at -70^{0}C and thawed to -20^{0}C before and during cutting on a cryostat. The slices varied in thickness from 6 to 10 microns, depending on the tissue type. The slices were transferred to SuperFrost Plus microslides (VWR Scientific, West Chester, PA), dried several hours at room temperature, and stored at -20^{0}C until use.

Preparation of DNA for generation of ^{33}P-labeled RNA. The pB1-BP7 construct was digested with SmaI endonuclease (antisense) and MspI endonuclease (sense) in separate reactions. The digestions were extracted with phenol and chloroform, ethanol precipitated, and resuspended in DEPC H_2O.

^{33}P-labeled riboprobes. Transcription reactions were performed in a solution containing 1 microgram DNA, 10X transcription buffer (Boehringer Mannheim Biochemicals, Germany), 10mM each of ATP, GTP, and CTP, alpha ^{33}P-UTP (Dupont-NEN, Maryland) and RNase Inhibitor (Boehringer Mannheim, Germany). T7 RNA Polymerase transcribed

the SmaI-digested DNA to yield a 600 nucleotide probe (antisense) and T3 RNA Polymerase transcribed the MspI to give a 200 nucleotide probe (sense - control).

In Situ Hybridization. The slides, containing various tissues, were fixed and delipidated. The slides were hybridized in the presence of the ^{33}P-labeled riboprobes described above, washed the following day, dipped in NTB-3 emulsion (Kodak), and stored between 4-8^{0}C.

Developing and staining. The slides were developed using D-19 developer (Kodak) and fixer (Kodak). Slides were developed at one, two, and four weeks after dipping in emulsion. Slides were stained with hematoxylin and eosin immediately after developing and dipped in xylenes before coverslipping with Cryoseal XYZ (Stephens Scientific, Riverdale, NJ). Those slides that were not stained were coverslipped using Aqua-mount (Lerner Laboratories, Pittsburgh, PA).

Results and Discussion

Northern analysis studies revealed the 1.5kb IGFBP-7 transcript to be highly expressed (+++) in kidney, strongly expressed (++) in lung, and expressed (+) in brain, heart, liver, and ovaries of C57BL6 mice. In kidney, a second transcript of IGFBP-7 at 3.1kb is expressed, suggesting that this may be a splice variant of the gene **[Figure 6]**.

RNA *in situ* hybridization studies using sense and antisense IGFBP-7 ^{33}P-labeled riboprobes were performed to determine cell specific expression within those tissues that demonstrated IGFBP-7 expression by Northern analysis. We include tissues that Kato *et al.* showed expression with the murine homologue of IGFBP-7 (12). Our studies have shown that there is some cell specificity of expression and that some tissues show a difference in level of expression between male and female mouse **[Figure 7]**. Expression is higher in female liver and brain.

In the brain, preliminary and subsequent studies using ICR and C57BL6 male and female mice show IGFBP-7 expression to be present in the hippocampus and cerebellum. Closer examination shows hybridization to be occurring in the cerebral cortex region, specifically to the granule cells, those cells that also predominately make up the hippocampus and the cerebellum. Expression appears to be higher in female brain in comparison to male brain.

Hybridization has been seen in the epithelial cells lining the ducts in mammary tissue of female mice. No hybridization was seen in the male counterpart, most likely due to a lack

of ducts in male mammary tissue. Female uterus showed IGFBP-7 hybridization of epithelial cells that line the lumen of the uterine horn. Studies on ovaries also suggest IGFBP-7 expression, although not to any particular cell type.

Conclusions

- IGFBP-7 has two RNA transcripts present in the kidney, indicating existence of splice variants.
- Differences of mRNA expression in various tissues is demonstrated between genders.

CONCLUSION

New Sequence

Research completed demonstrates that PSF, TAF, and IGFBP-7, whose sequences show 98.9% identity, are the same conservative differences in amino acid sequence can be explained by polymorphism (BCM Search Launcher, Netscape). **[Figure 3]**

Retroviral Vector

To study the potential of IGFBP-7 as a modulator of cell growth, retroviral vectors containing the sense and antisense forms of IGFBP-7 were created. These vectors were used to transfect the ectropic cell line PE501 and generate virus. The virus was used to infect PA317 to package the virus for infection of human cell lines. Before using the virus made by PA317 cells to infect the cell lines of interest, PA317 clones were checked for unrearranged vector, titer, and expression of the inserted gene.

Southern blots, containing genomic DNA collected from 10 PA317 clones (5 sense, 5 antisense) that was digested with SmaI overnight, were probed with IGFBP-7 (865bp) and LXSN (1.67kb, 3'end). Results reveal no rearrangement of the vector **[Figure 3]**. Northern analysis of RNA collected from the above clones demonstrated expression of both indigenous and retroviral IGFBP-7 **[Figure 1]**.

Promoter Studies

Ten clones have been isolated from an ATCC genomic library enriched fro chromosome 4 (IGFBP-7 mapped by FISH (6)). Four of the ten clones have been subjected to restriction digests and PCR analysis using various primers within the IGFBP-7 sequence and primers flanking the library insert site on Charon 21A. Preliminary results suggest that we have

one clone with 800 base pairs 5' of IGFBP-7 and another clone with approximately 3.6 kilobases of sequence 5' of IGFBP-7 **[Figure 4]**. Further studies will be done to determine if any promoter region is contained, as well as subjecting the other six clones to the same analysis.

Characteristics

Westerns blots, using crosslinked and uncrosslinked conditioned medium collected from normal (AG11132) and breast cancer (Hs578T, MCF7) and Anti-BP7 antibodies, demonstrate a band at approximately 50 kDa in uncrosslinked Hs578T and hR IGFBP-7. This experiment demonstrates: 1) the protein needed to be concentrated to allow detection and 2) we have access to an antibody that recognizes native protein.

A band was not detected in AG11132 (passage 10) crosslinked and uncrosslinked sample, nor was it found in crosslinked Hs578T **[Figure 5]**. The lack of bands in AG11132 could be a result of collecting conditioned medium at an early passage. Previous work show IGFBP-7 expression to be upregulated in senescent cells (6). AG11132 cultures are currently being grown and conditioned medium collected, starting with passage 15. Senescence-associated endogenous ß-galactosidase activity is being measured at each passage to detect the percentage of senescent cells present.

Absence of a band in the crosslinked Hs578T conditioned medium may be caused by crosslinking occurring in the region responsible for antibody recognition. A different crosslinker which has reversible properties will be tested. The reversible crosslinker offers several advantages: first, it may cause crosslinking to occur in a region of the protein that is not necessary for antibody binding; second, if crosslinking should still occur in the antibody binding region, a Western blot on uncrosslinked, crosslinked, and decrosslinked samples should show binding on the uncrosslinked and decrosslinked samples. This would indicate that IGFBP-7 is binding a protein/ligand *in vitro.*

The importance of this experiment is 1) we may determine if IGFBP-7 binds other proteins, in addition to IGF-I and IGF-II, and 2) it demonstrates that we now have an antibody that can be used in other studies (*i.e. In situ* immunohistochemistry to determine protein localization).

Expression/Specificity

Northern analysis demonstrates IGFBP-7 mRNA expression present in all tissues tested, although levels vary between tissues **[Figure 6]**. We found two mRNA transcripts in kidney which demonstrates the highest level of IGFBP-7 expression of the tissues examined. This indicates that IGFBP-7 has splice variants.

To understand exactly where expression was occurring, especially in organs that are highly complex (i.e. brain), in situ hybridization using ^{33}P-labeled riboprobes and various tissues collected from male and female C57BL6 mice was undertaken.

We observe specific IGFBP-7 expression by RNA *in situ* hybridization in the hippocampus, cerebellum, and the cortex. In these regions of the brain hybridization is occurring over the granule cells. In the cerebellum, hybridization seen in the granular and Purkinje layers. Both cell types are projection neurons. The granule cells in this region are cerebellar interneurons. The Purkinje cells are inhibitory projection neurons and are part of one of the strongest excitatory connections in the central nervous system. Expression appears to be higher in the female than in the male. Other findings suggest this difference to include the liver and spleen, which also demonstrate a higher level of expression in female tissue. Ovary, mammary tissue, and uterus demonstrate IGFBP-7 mRNA expression, especially in the intralobular ducts (MT), uterine glands (U), and simple columnar epithelium (U).

Summary

We are beginning to understand the potential functions of IGFBP-7. Not only is it associated with an IGF-like function, it has also been linked to other functions: a tumor-derived adhesion factor and a prostacyclin-stimulating factor. The weak binding of IGFBP-7 to IGFs suggests that it may control/play a role in growth control by a different mechanism. Because IGFBP-7 appears to be expressed in all tissues examined but at varying levels, it is important to pinpoint which cells are expressing mRNA and if expression at the RNA level mirrors protein levels. Retroviral studies currently underway in our lab should greatly aid in determining what type of biological effect mac25 is having on cell growth in an *in vitro* system.

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[redacted]

A.

```
              1             15 16            30 31            45 46            60 61            75 76            90
1 IGFBP-7     ---------CTCTAA AGCCGCCATGGAGCG GCCGTCGCTGCGCGC CCTGCTCCTCGGCGC CGCTGGGCTGCTGC- TCCTGCTCCTGCCCC   80
2 mac25       ---------CTCTAA AGCCGCCATGGAGCG CGCGTCGCTGCGCGC CCTGCTCTTCGGCCC CGCTGGGCTGCTGC- TCCTGCTCCTGCCCC   80
3 PSF         GCCGCTGCCACCGCA CCCCGCCATGGAGCG GCCGTCGCTGCGCGC CCTGCTCCTCGGCGC CGCTGGGCTGCTGC- TCCTGCTCCTGCCCC   89
4 follistatin --------------- --------------- ----ATGGTCTGCGC CAGGCAC--CAGCCC GGC-GGGCT-CTGCC TCCTGCTGCTGCTAC   52
              91           105 106          120 121          135 136          150 151          165 166          180
1 IGFBP-7     TCT-CCTCTTCCTCC TCTTCGGACACCTGC GGCCC--CTGCGA-- -GCCGGCCTCCTGCC CGCCCCTGCCC--CC GCTGGGCTG-CCTGC  161
2 mac25       TCT-CCTCTTCCTCC TCTTCGGACACCTGC GGCCC--CTGCGA-- -GCCGGCCTCCTGCC CGCCCCTGCCC--CC GCTGGGCTG-CCTGC  161
3 PSF         TCT-CCTCTTCCTCC TCTTCGGACACCTGC GGCCC--CTGCGA-- -GCCGGCCTCCTGCC CGCCCCTGCCC--CC GCTGGGCTG-CCTGC  170
4 follistatin TCTGCCAGTTCAT-- --GGAGGACCGCAGC GCCCAGGCTGGGAAT TGCTGGC-TCC-GCC AAGCAAAGAACGGCC GCTGCCAGGTCCTGT  136
              181          195 196          210 211          225 226          240 241          255 256          270
1 IGFBP-7     -TGGGCGAGACCCGC G-----ACGCGTGCG GCTGCT---GCCCTA TGTGCGCC-CGCGG- -CGAGGGCGAGCCGT GCGGGGGTGGCGGCG  239
2 mac25       -TGGGCGAGACCCGC G-----ACGCGTGCG GCTGCT---GCCCTA TGTGCGCC-CGCGG- -CGAGGGCGAGCCGT GCGGGGGTGGCGGCG  239
3 PSF         -TGGGCGAGACCCGC G-----ACGCGTGCG GCTGCT---GCCCTA TGTGCGCC-CGCGG- -CGAGGGCGAGCCGT GCGGGGGTGGCGGCG  248
4 follistatin ATAAGACAGAACTGA GCAAGGAAGAGTGTT GCAGCACCGGCCGGC TGAGCACCTCATGGA CCGAGGAGGATGTGA ACGACAATACTCTCT  226
              271          285 286          300 301          315 316          330 331          345 346          360
1 IGFBP-7     CCGGCAGGGGG---T ACTGCGCGCCGGGCA TGGAGTGC------- GTGAAGAGCCGCAAG AAGCGGAAGGGTAAA G-CCGGGGCAGCA--  316
2 mac25       CCGGCAGGGGG---T ACTGCGCGCCGGGCA TGGAGTGC------- GTGAAGAGCCGCAAG AGGCGGAGGGGTAAA G-CCGGGGCAGCA--  316
3 PSF         CCGGCAGGGGG---T ACTGCGCGCCGGGCA TGGAGTGC------- GTGAAGAGCCGCAAG AGGCGGAAGGGTAAA G-CCGGGGCAGCA--  325
4 follistatin TCAAGTGGATGATTT TCAACGGGGGCGCCC CCAACTGCATCCCTT GTAAAGAAACGTGTG AGAACGTGGACTGCG GACCTGGGAAAAAAT  316
              361          375 376          390 391          405 406          420 421          435 436          450
1 IGFBP-7     GCCGGCGGTCCGGGT TTAA--------GCG GCGTGTGCG------ --TGTGC-AAGAGCC GCTA------CCCGG TGTGCGGCAGCGACG  383
2 mac25       GCCGGCGGTCCGGGT GTAA--------GCG GCGTGTGCG------ --TGTGC-AAGAGCC GCTA------CCCGG TGTGCGGCAGCGACG  383
3 PSF         GCCGGCGGTCCGGGT GTAA--------GCG GCGTGTGCG------ --TGTGC-AAGAGCC GCTA------CCCGG TGTGCGGCAGCGACG  392
4 follistatin GTCGAATGAACAAGA AGAATAAACCCCGCT GCGTCTGTGCCCCAG ACTGTTCCAACATCA CCTGGAAGGGCCCAG TGTGTGGGCTGGATG  406
              451          465 466          480 481          495 496          510 511          525 526          540
1 IGFBP-7     GCACCACCTACCCGA GCGGCTGCCAGCTGC GCGCCGCCAG-CCAG AGGGCCGAGAGCCGC GGGGA---GAAGGCC ATCACCCAGGTCA--  467
2 mac25       GCACCACCTACCCGA GCGGCTGCCAGCTGC GCGCCGCCAG-CCAG AGGGCCGAGAGCCGC GGGGA---GAAGGCC ATCACCCAGGTCA--  467
3 PSF         GCACCACCTACCCGA GCGGCTGCCAGCTGC GCGCCGCCAG-CCAG AGGGCCGAGAGCCGC GGGGA---GAAGGCC ATCACCCAGGTCA--  476
4 follistatin GGAAAACCTACCGCA ACGAATG-----TGC ACTCCTCAAGGCCAG ATG-CAAAGAGCAGC CGGAACTAGAAGTAC AGTACC-AGGCAAA  489
              541          555 556          570 571          585 586          600 601          615 616          630
1 IGFBP-7     -GCAAGGGCACCTGC GAGCAAGGTCCTTCC ATAGTGACGCCCCCC AAGGACATCTGGAAT GTCACTGGTGCCCAG GTGTACTTGAGCTGT  556
2 mac25       -GCAAGGGCACCTGC GAGCAAGGTCCTTCC ATAGTGACGCCCCCC AAGGACATCTGGAAT GTCACTGGTGCCCAG GTGTACTTGAGCTGT  556
3 PSF         -GCAAGGGCACCTGC GAGCAAGGTCCTTCC ATAGTGACGCCCCCC AAGGACATCTGGAAT GTCACTGGTGCCCAG GTGTACTTGAGCTGT  565
4 follistatin TGTAAAAAGACTTGT AGGGATGTTTTTTGT CCAG-GCAGCTCCAC TTG----TGTGGTGG ATCA-----GACCAA TAATGCCTACTGTGT  569
              631          645 646          660 661          675 676          690 691          705 706          720
1 IGFBP-7     GAG--GTCATCGGAA TC-CCGACACCTGTC TTCATCTGGAACAAG GTAAAAAGGGGTCAC T-ATGGAGTT-CAAA GGACAGAACTCCTGC  641
2 mac25       GAG--GTCATCGGAA TC-CCGACACCTGTC CTCATCTGGAACAAG GTAAAAAGGGGTCAC T-ATGGAGTT-CAAA GGACAGAACTCCTGC  641
3 PSF         GAG--GTCATCGGAA TC-CCGACACCTGTC CTCATCTGGAACAAG GTAAAAAGGGGTCAC T-ATGGAGTT-CAAA GGACAGAACTCCTGC  650
4 follistatin GACCTGTAATCGGAT TTGCCCAGAGCCCTC CTCTTCTG-AACA-- GTACCTTTGTGGAAA TGATGGAGTGACTTA CTCCAG--CGCCTGC  654
              721          735 736          750 751          765 766          780 781          795 796          810
1 IGFBP-7     CTGGTGACCGGGACA ACCTG---GCCATTC AGACCC---GGGGTG GCCCAGAAAAGCATG AAGTAACTGGCTGGG TGCTGGTATCTCCTC  725
2 mac25       CTGGTGACCGGGACA ACCTG---GCCATTC AGACCC---GGGGTG GCCCAGAAAAGCATG AAGTAACTGGCTGGG TGCTGGTATCTCCTC  725
3 PSF         CTGGTGACCGGGACA ACCTG---GCCATTC AGACCC---GGGGTG GCCCAGAAAAGCATG AAGTAACTGGCTGGG TGCTGGTATCTCCTC  734
4 follistatin CACCTGAGAAAGGCC ACCTGCTTGCTGGGC AGATCCATTGGATTA GCCTATGAGGG---A AAGTGTATCACAAAG TCCTGTGAAGATATC  741
              811          825 826          840 841          855 856          870 871          885 886          900
1 IGFBP-7     TAAGTAAGGAAGATG CTGGAGAATATGAGT GCCATGCATCCAATT CCCAAGGACAGGCTT CAGCATCAGCAAAAA TTACAGTG-GTTGAT  814
2 mac25       TAAGTAAGGAAGATG CTGGAGAATATGAGT GCCATGCATCCAATT CCCAAGGACAGGCTT CAGCATCAGCAAAAA TTACAGTG-GTTGAT  814
3 PSF         TAAGTAAGGAAGATG CTGGAGAATATGAGT GCCATGCATCCAATT CCCAAGGACAGGCTT CAGCATCAGCAAAAA TTACAGTG-GTTGAT  823
4 follistatin CA-GTGTGGCGG--- CGGGAAAAAATGCCT -------ATGGGATT CC-AAGGTT-GGCAG AGGTCGCTGCTCTCT CTGCGATGAGCTGTG  818
              901          915 916          930 931          945 946          960 961          975 976          990
1 IGFBP-7     GCCTTACATGAAATA -CCAGTGAAAAAAGG TGAAGGTGCCGAGCT ATAA
2 mac25       GCCTTACATGAAATA GCCAGTGAAAAAAGG TGA
3 PSF         GCCTTACATGAAATA -CCAGTGAAAAAAGG TGAAGGTGCCGAGCT ATAA
4 follistatin TCCTGACAGTAAGT- -CGGATGAGC--CGG TCT--GTGCC-AGTG ACAATGCCACATACG CCAGCGAGTGTGCCA TGAAGGAAGCTG-CC  900
              991         1005 1006        1020 1021        1035 1036        1050 1051        1065 1066        1080
4 follistatin TGCTCTTCTGGCGTG CTTCTTGAAGTGAAG CATTCTGGATCTTGC AACTCCATCTCGGAA GAAACGGAGGAAGAG GAGGAGGAGGAAGAC  990
              1081        1095 1096        1110 1111        1125 1126        1140 1141        1155 1156        1170
4 follistatin C-AGGACTACAGCTT TCCTATCTCTTCCAT TCTAGAGTGGTAA
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B.

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              1             15 16            30 31            45 46            60 61            75 76            90
1 IGFBP-7     -----------MERP SLRALLLGAAGLLLL LLPLSSSSSSD---- ----TCGPCEP---- -ASCPPL-------- --PPLGCLLG----E   52
2 PSF         -----------MERP SLRALLLGAAGLLLL LLPLSSSSSSD---- ----TCGPCEP---- -ASCPPL-------- --PPLGCLLG----E   52
3 mac25       -----------MERA SLRALLFGPAGLLLL LLPLSSSSSSD---- ----TCGPCEP---- -ASCPPL-------- --PPLGCLLG----E   52
4 follistatin MGAAAAQAPLGLPAA SARLLLLATSVLLLF AFSLPGSRASNQPPG GGGGTGGDCPGGKGK SINCSELNVRESDVR VCDESSCKYGGVCKE   90

              91           105 106          120 121          135 136          150 151          165 166          180
1 IGFBP-7     TRD--ACGCCPMCAR GEGEPCGGGG----- ---AGRGYCAPGME- -------CVKSRKKR RGK-----AGAAG- -----GP--------  105
2 PSF         TRD--ACGCCPMCAR GEGEPCGGGG----- ---AGRGYCAPGME- -------CVKSRKRR KGK-----AGAAG- -----GP--------  105
3 mac25       TRD--ACGCCPMCAR GEGEPCGGGG----- ---AGRGYCAPGME- -------CVKSRKRR RGK-----AGAAG- -----GP--------  105
4 follistatin DGDGLKCACQFQCHT NYIPVCGSNGDTYQN ECFLRRAACKHQKEI TVIARGPCYSDNGSG SGEGEEEGSGAEVHR KHSKCGPCKYKAECD  180

              181          195 196          210 211          225 226          240 241          255 256          270
1 IGFBP-7     -GVSGV-CVCK---S RY---PVCGSDGTTY PSGCQLRAASQRAES RGEKAITQVSKGTCE Q--GPSIVTPPKD-- IW--NVTGAQVYLSC  181
2 PSF         -GVSGV-CVCK---S RY---PVCGSDGTTY PSGCQLRAASQRAES RGEKAITQVSKGTCE Q--GPSIVTPPKD-- IW--NVTGAQVYLSC  181
3 mac25       -GVSGV-CVCK---S RY---PVCGSDGTTY PSGCQLRAASQRAES RGEKAITQVSKGTCE Q--GPSIVTPPKD-- IW--NVTGAQVYLSC  181
4 follistatin EDAENVGCVCNIDCS GYSFNPVCASDGSSY NNPCFVREASCIKQ- --E-QIDIRHLGHCT DTDDTSLLGKKDDGL QYRPDVKDASDQRED  266

              271          285 286          300 301          315 316          330 331          345 346          360
1 IGFBP-7     EVIGIPTPVFIWNKV KRGHYGVQRTELLPG DRDNLAIQTRGGPEK HEVTGWVLVSPLSKE DAGEYECHASNSQGQ ASASAKIT--VVDAL  269
2 PSF         EVIGIPTPVLIWNKV KRGHYGVQRTELLPG DRDNLAIQTRGGPEK HEVTGWVLVSPLSKE DAGEYECHASNSQGQ ASASAKIT--VVDAL  269
3 mac25       EVIGIPTPVLIWNKV KRGHYGVQRTELLPG DRDNLAIQTRGGPEK HEVTGWVLVSPLSKE DAGEYECHASNSQGQ ASASAKIT--VVDAL  269
4 follistatin VYIGNHMPCPENLNG YCIHGKCEFIYLLRR ASCRCESGYTGQHCE KTDFSILYVVP-SRQ KLTHVLIAAIIGAVQ IAIIVAIVMCITRKC  355

              361          375 376          390 391          405 406          420 421          435 436          450
1 IGFBP-7     HEIPVKKGEGAEL-- ----------      282
2 PSF         HEIPVKKGEGAEL-- ----------      282
3 mac25       HEIASEKR------- ----------      277
4 follistatin PKNNRGRRQKQNLGH FTSDTSSRMV      380
```

Figure 2. Nucleotide (A) and amino acid (B) alignment of IGFBP-7, mac25, PSF, and follistatin using ClustalW Multiple Sequence Alignment through BCM Search Launcher on Netscape. Underline (___) = stop codon, dashes (---) = gaps in sequence.

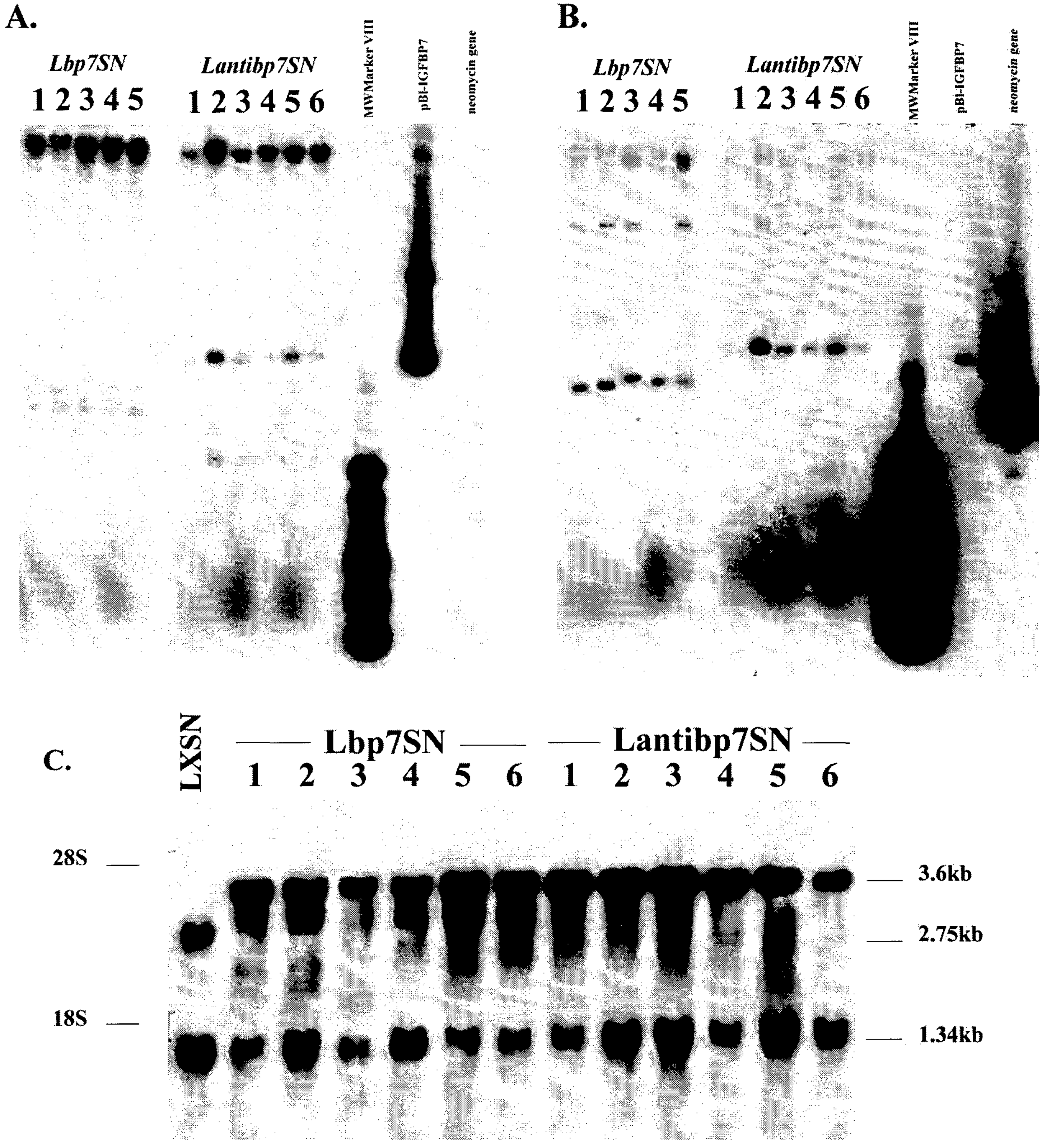
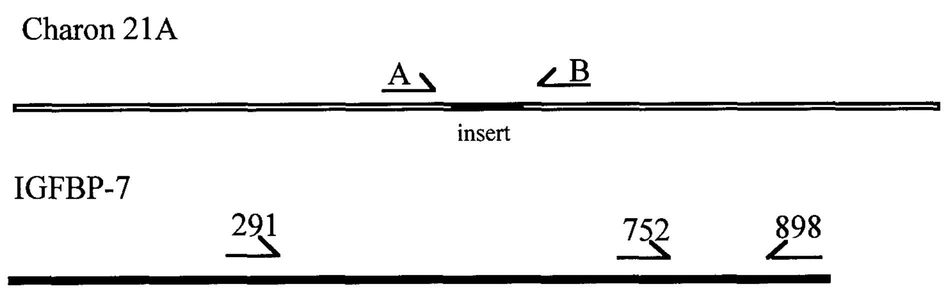


Figure 3. **Characterization of retroviral vector containing sense and antisense IGFBP-7.** Ten micrograms of genomic DNA was digested with SmaI, the bands separated by gel electrophoresis, and transferred to Zeta Blot. A. Blot probed with radiolabeled IGFBP-7. B. Blot probed with radiolabeled neomycin gene.

Ten micrograms of total RNA was collected from PA317, the bands separated by gel electrophoresis, and transferred to Zeta Blot. Blot probed with radiolabeled IGFBP-7 not shown. C. Blot probed with radiolabeled neomycin gene.



Primer Combinations Tested:

A and B
A and 291
B and 291
A and 752
B and 752
A and 898
B and 898

Results:

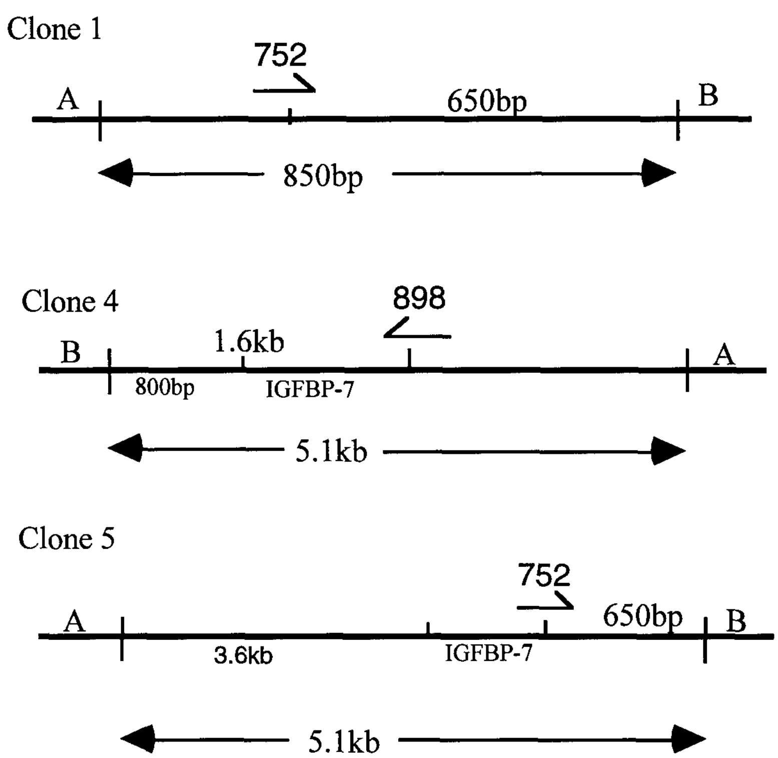


Figure 4. **Model of PCR products generated from genomic lambda phage.** A genomic library, enriched for chromosome 4 and inserted at the HindIII restriction in the Charon 21A, was screened with ^{32}P-labeled IGFBP-7. Ten candidate clones were found. Primers A and B flank this region of Charon 21A. Three primers within the IGFBP-7 were designed and used in various combinations with primers A and B to screen four of the clones for insert size and orientation. The double-headed arrows denote the total length of the recovered insert.

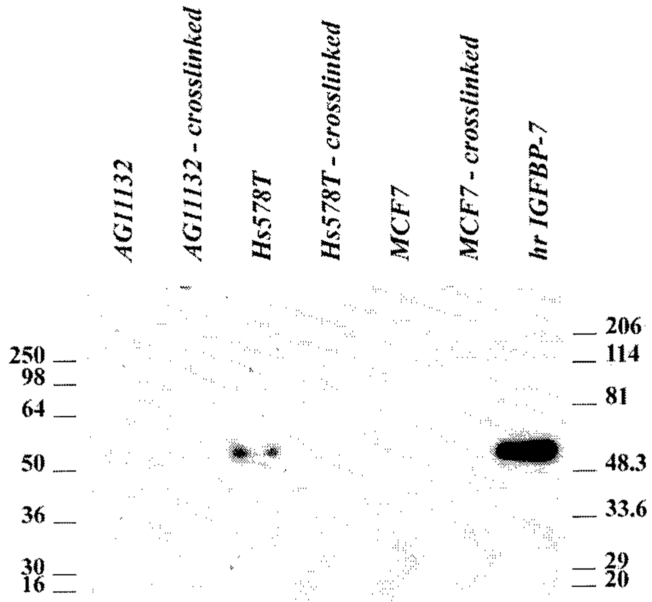


Figure 5.

Western Blot Analysis. Conditioned medium was collected from AG11132 (passage 10), Hs578T, and MCF7 cells. Half of the medium was crosslinked using dissucinyl suberate (DSS). Proteins in both crosslinked and uncrosslinked conditioned medium was precipitated with ammonium sulfate and dialyzed in 10% glycerol/PBS. Final volume was 500 microliters (concentrated from 54 ml). Ten microliters was mixed with 10 microliters of 2X Sample Buffer containing ß-mercaptoethanol, separated on a 3%/12% polyacrylamide gel, and transferred to nitrocellulose. The membrane was blocked and incubated with primary anti-IGFBP7 and secondary donkey anti-rabbit HPR antibodies. Enhanced chemiluminescence allowed detection of primary antibody binding.

Mentor: Karen L. Swisshelm, Ph.D.
Trainee: Heather-Marie P. Wilson

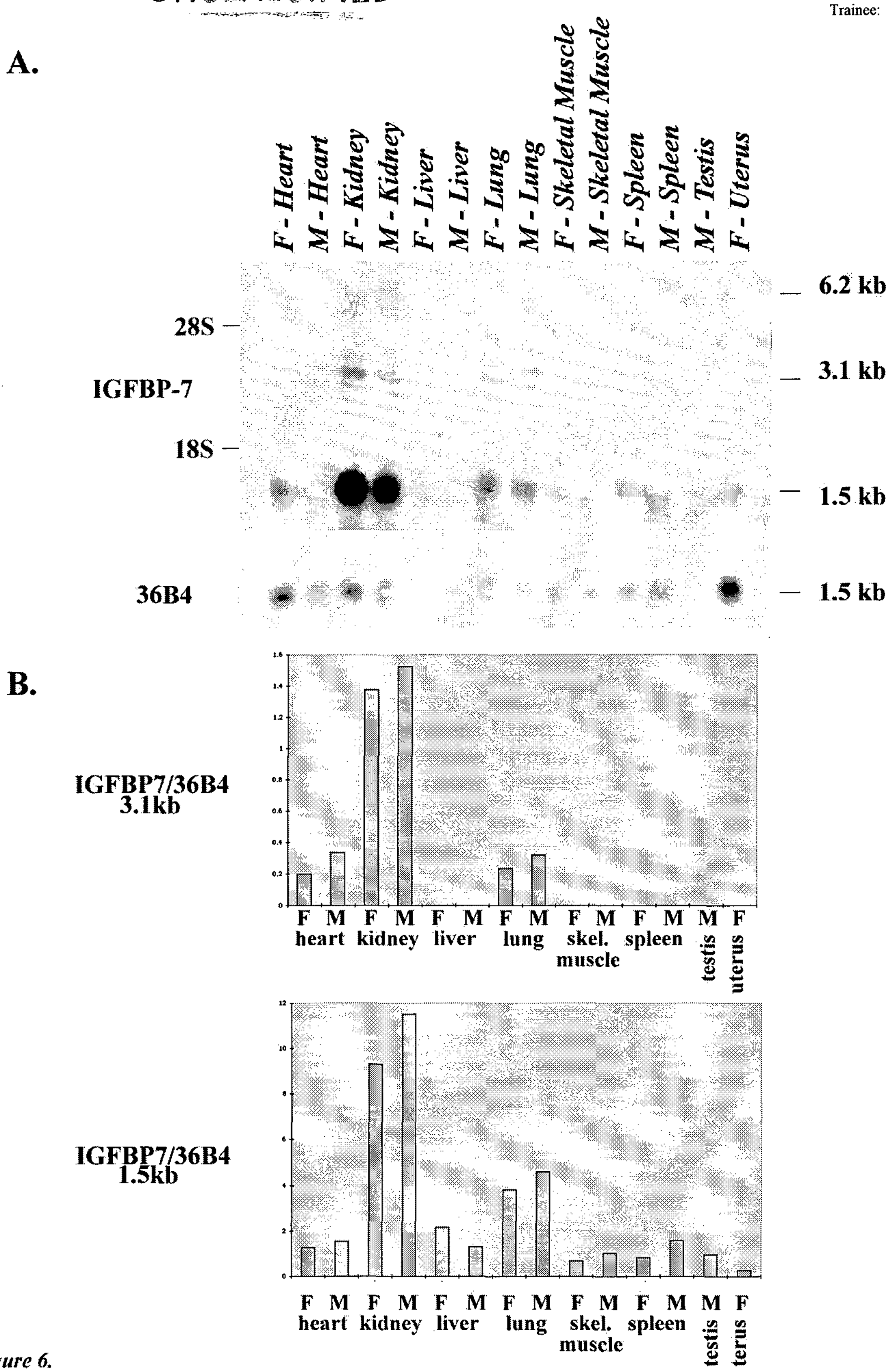


Figure 6.

A. Northern Analysis. Ten micrograms of total RNA from tissues of C57BL6 male and female mice was subjected to gel electrophoresis and transferred to Zeta Blot (BIORAD). The membrane was probed with IGFBP-7 cDNA, then stripped and reprobed with 36B4 for loading control.

B. Densitometry. Densitometry was done by NIH Image 1.6 and interpreted by graphs generated in Excel 5.1. The X axis denotes tissues and animal gender. The Y axis shows the IGFBP-7/36B4 ratio acquired from the densitometry.

Mentor: Karen L. Swisshelm, Ph.D.
Trainee: Heather-Marie P. Wilson

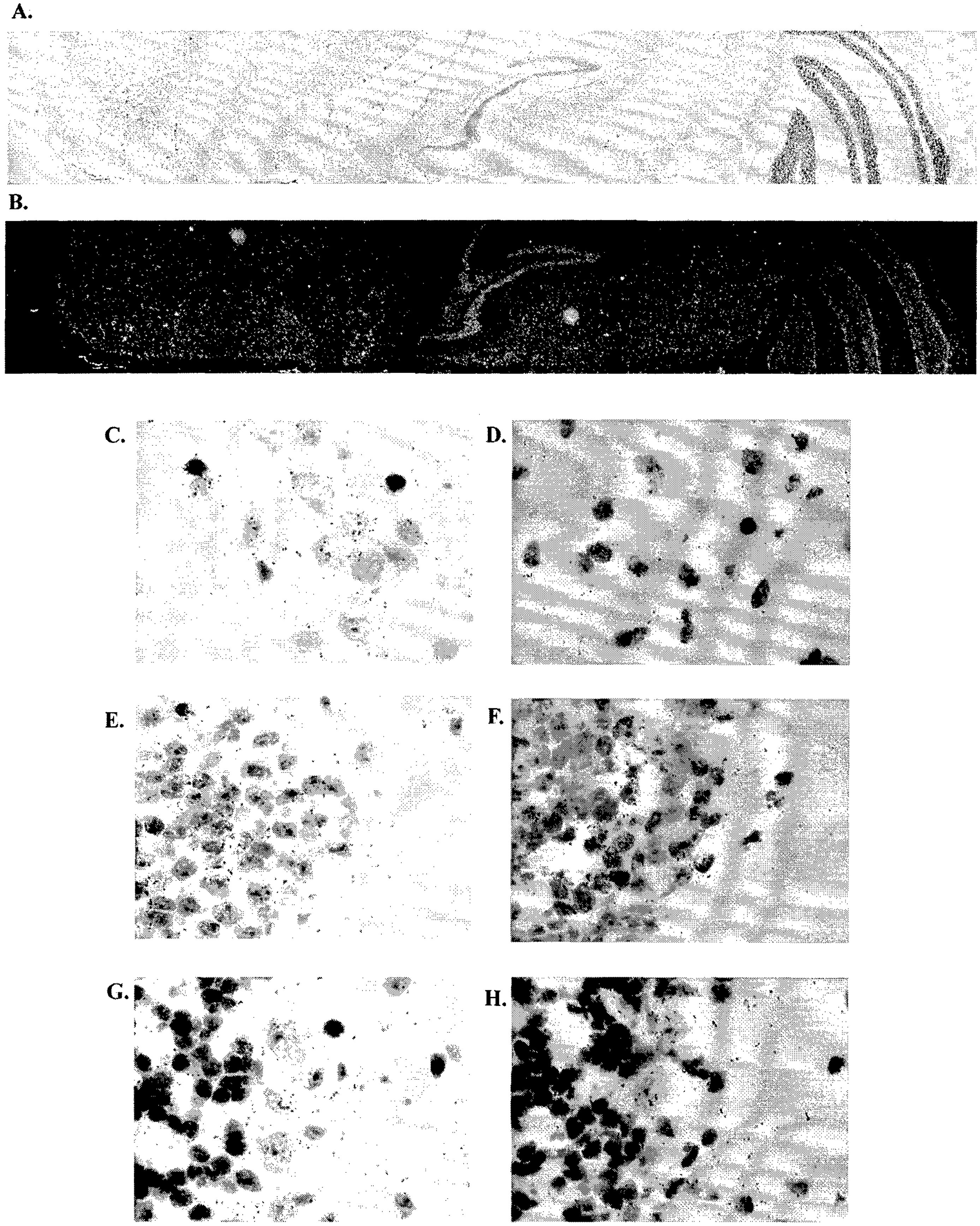


Figure 7. **In situ hybridization on male and female C57Bl6 mouse brain.** A. 4X, female (F), bright field (BF). B. 4X, F, dark field (DF). C. 100X, F, BF, cortex. D. 100X, male (M), BF,cortex. E. 100X, F, BF, hippocampus. F. 100X, M, BF, hippocampus. G. 100X, F, BF, cerebellum. H. 100X, M, BF, cerebellum.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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REPLY TO
ATTENTION OF:

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ADB251995	ADB233334	ADB237451	
ADB233106	ADB242926	ADB249671	
ADB262619	ADB262637	ADB262475	
ADB233111	ADB251649	ADB264579	
ADB240497	ADB264549	ADB244768	
ADB257618	ADB248354	ADB258553	
ADB240496	ADB258768	ADB244278	
ADB233747	ADB247842	ADB257305	
ADB240160	ADB264611	ADB245442	
ADB258646	ADB244931	ADB256780	
ADB264626	ADB263444	ADB264797	